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Role of Jasmonates in the Elicitor- and Wound-Inducible Expression of Defense Genes in Parsley and Transgenic Tobacco[1]

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Jasmonates have been proposed to be signaling intermediates in the wound and/or elicitor-activated expression of plant defense genes. We used parsley (*Petroselinum crispum*) cell cultures and transgenic tobacco (*Nicotiana tabacum*) plants expressing *4CL1-GUS* gene fusions to investigate the potential role played by jasmonates in mediating the wound and/or elicitor activation of phenylpropanoid and other defense-related genes. Jasmonates and α-linolenic acid strongly induced the expression of *4CL* in a dose-dependent manner in parsley cells; methyl jasmonate also activated the coordinate expression of other phenylpropanoid genes and the accumulation of furanocoumarin phytoalexins. However, the response of the cells to optimal methyl jasmonate concentrations was distinct quantitatively and qualitatively from the response of elicitor-treated cells. In transgenic tobacco wound-inducible tobacco *4CL* genes and a *4CL1* promoter-*GUS* transgene were responsive to jasmonates and α-linolenic acid in a dose-dependent manner. Pretreatment of parsley cells or tobacco leaves with a lipoxygenase inhibitor reduced their responsiveness to the elicitor and to wounding. These results show that the elicitor response in parsley cells can be partially mimicked by jasmonate treatment, which supports a role for jasmonates in mediating wound-induced expression of *4CL* and other phenylpropanoid genes.

The stimulation of phenylpropanoid metabolism is an important plant defense against environmental stresses such as wounding or pathogen attack. Increased carbon flow through this pathway leads to the accumulation of compounds with roles in structural defense (e.g. lignin and other wall-bound phenolics) and those with fungitoxic properties (e.g. isoflavonoid and furanocoumarin phytoalexins) (Hahlbrock and Scheel, 1989). PAL, the first enzyme of the general phenylpropanoid pathway, catalyzes the deamination of Phe to yield cinnamic acid and thus plays a key role in diverting photosynthate from primary metabolism into phenylpropanoid metabolism. The third and final step in the general phenylpropanoid pathway, catalyzed by 4CL, leads to the activation of substituted cinnamic acids by the formation of hydroxycinnamoyl:CoA esters. These CoA esters are used as substrates for a variety of branch pathways specific for the biosynthesis of particular secondary compounds such as flavonoids and lignin monomers.

The activation of phenylpropanoid metabolism in response to pathogen challenge and wounding has been demonstrated in a number of plants (reviewed by Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). In parsley cell cultures *PAL* and *4CL* genes are transcriptionally activated in a coordinate manner by UV light irradiation or treatment with Pmg (Hahlbrock and Scheel, 1989). Transcriptional activation of genes encoding enzymes specific to flavonoid biosynthesis (e.g. CHS) and furanocoumarin biosynthesis (e.g. BMT) is signal specific: elicitor treatment activates transcription of *BMT* and furanocoumarin accumulation in culture filtrates (Hauffe et al., 1986; Hauffe, 1988; Hahlbrock and Scheel, 1989), and UV light activates transcription of *CHS* and vacuolar flavonoid accumulation (Matern et al., 1983; Chappell and Hahlbrock, 1984). In parsley (*Petroselinum crispum*) plants *PAL* and *4CL* expression is activated locally in response to wounding and pathogen infection and is developmentally regulated (Schmelzer et al., 1989; Wu and Hahlbrock, 1992). In transgenic tobacco the parsley *4CL1* promoter fragment directs wound-inducible *GUS* reporter gene expression and complex patterns of developmentally regulated *GUS* expression in a manner consistent with *4CL* mRNA accumulation assayed by in situ hybridization (Douglas et al., 1991; Hauffe et al., 1991; Wu and Hahlbrock, 1992; Reinold et al., 1993).

In addition to the activation of phenylpropanoid gene expression, the transcriptional induction of a set of non-phenylpropanoid defense-related genes is characteristic of the elicitor response in parsley cells. Functions for some of these *ELI* genes, which were originally identified solely on the basis of their elicitor responsiveness (Somssich et al., 1989), have been subsequently assigned based on DNA sequence homologies and, in some cases, on functional tests and include Tyr decarboxylase (Kawalleck et al., 1993a) and HRGP (Trezzini et al., 1993).

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Abbreviations: BMT, bergaptol methyltransferase; CHS, chalcone synthase; 4CL, 4-coumarate:CoA ligase; HRGP, Hyp-rich glycoprotein; JA, jasmonic acid; LA, α-linolenic acid; MJ, methyl jasmonate; nPG, *n*-propyl gallate; PAL, Phe ammonia-lyase; *pin*, proteinase inhibitor gene; Pmg, elicitor preparation from *Phytophthora megasperma* f. sp. *glycinea*; *vsp*, vegetative storage protein gene.

The nature of intracellular signals that culminate in the stress-induced transcriptional activation of *4CL1* and other defense-related genes in parsley are not well understood, but the outlines of signaling events triggered by elicitor perception in parsley cells have recently begun to emerge. In parsley full Pmg activity resides in a glycoprotein purified from mycelial cell walls (Parker et al., 1991). Specific, reversible binding of an oligopeptide derived from this protein to sites on parsley plasma membranes activates *4CL* and other defense gene expression, as well as furanocoumarin accumulation in protoplasts (Nürnberger et al., 1994). Peptide binding also alters the permeability of the plasma membrane to Ca^{2+}, H^+, K^+, and Cl^- and induces H_2O_2 formation (Nürnberger et al., 1994). Elicitor-activated Ca^{2+} influx and protein phosphorylation (Dietrich et al., 1990) are thought to be part of a signaling cascade leading to defense gene activation (Nürnberger et al., 1994). In contrast, virtually nothing is known about the nature of wound-generated signals that lead to the activation of phenylpropanoid gene expression in parsley.

Recently, attention has focused on the potential role played by JA or its methyl ester MJ (referred to collectively as jasmonates) as intracellular signaling molecules that mediate the activation of gene expression in response to wounding, elicitor treatment, and pathogen infection (Anderson, 1989; Staswick, 1992; Sembdner and Parthier, 1993; Farmer, 1994; Reinbothe et al., 1994; Blechert et al., 1995; Creelman and Mullet, 1995). Farmer and Ryan (1992) proposed that the activation of *pin* gene expression by mechanical wounding is mediated by JA. They hypothesized that perception of a local or systemic wound-generated signal at the cell surface is followed by the release of LA from membranes, which is then converted to JA via a biosynthetic pathway involving the action of a lipoxygenase followed by dehydration, reduction, and several β-oxidation steps (Vick and Zimmerman, 1984). Consistent with this hypothesis, expression of *pin* genes is activated by MJ, JA, LA, and octadecanoid precursors of JA in tomato and potato (Farmer and Ryan, 1992; Farmer et al., 1992; Hildmann et al., 1992; Peña-Cortés et al., 1993), and the expression of wound-inducible *vsp* genes is activated by jasmonates in soybean (Staswick et al., 1991; Creelman et al., 1992). Furthermore, JA and MJ accumulate in wounded soybean and tomato tissues (Creelman et al., 1992; Peña-Cortés et al., 1993), and inhibitors of lipoxygenase and other enzymatic steps required for JA biosynthesis inhibit wound-induced *pin2* mRNA accumulation in tomato (Peña-Cortés et al., 1993) and *vsp* mRNA accumulation in soybean (Staswick et al., 1991).

The role played by jasmonates in mediating the wound- and/or elicitor-activated expression of phenylpropanoid and defense-related genes other than *pin* genes has not been thoroughly explored, despite suggestions that jasmonates are key signaling intermediates in these responses (Dittrich et al., 1992; Mueller et al., 1993). Suspension-cultured cells from a number of plants rapidly accumulate JA and jasmonate precursors in response to treatment with a yeast cell-wall elicitor (Mueller et al., 1993), and secondary metabolite accumulation and, in the case of soybean cells, *PAL* and *CHS* gene expression is activated by treatment with exogenous jasmonate (Creelman et al., 1992; Gundlach et al., 1992). Exogenously added phytodienoic acid, a jasmonate precursor, was reported to weakly activate *PAL*, *4CL*, and *CHS* mRNA accumulation in parsley cells (2- to 5-fold above controls; Dittrich et al., 1992), and JA was reported to weakly activate furanocoumarin accumulation in suspension-cultured parsley cells (Kauss et al., 1992). In this study we used the parsley cell culture system and transgenic tobacco plants expressing *4CL1-GUS* gene fusions to better define the potential role played by jasmonates as intracellular signaling molecules that mediate the wound and/or elicitor activation of *4CL* and other parsley defense-related genes.

MATERIALS AND METHODS

A parsley (*Petroselinum crispum*) cell culture was provided by Dierk Scheel (Max-Planck Institute, Cologne, Germany) and propagated as described previously (Ragg et al., 1981). Transgenic tobacco lines containing either 597- or 210-bp parsley *4CL1* promoter-*GUS* fusions were derived from primary transformants previously described (Douglas et al., 1991; Hauffe et al., 1991) by selfing. Kanamycin-resistant F_1 progeny were selected by germinating seeds on Murashige-Skoog medium (GIBCO) supplemented with 100 μg/mL kanamycin and were subsequently propagated in soil in growth chambers. Parsley plants were grown from seed and propagated in the greenhouse.

Elicitor, Jasmonate, and Wounding Treatments

Crude Pmg elicitor, which was prepared from mycelia of *Phytophthora megasperma* f. sp. *glycinea* as described by Ayers et al. (1976) or was a gift of D. Scheel, was applied to dark-grown cell cultures at a final concentration of 50 μg/mL 5 d after subculture. MJ (Bedoukian Research, Danbury, CT) and JA (Apex Organics, Honiton, Devon, UK) stock solutions were prepared in 1% Triton X-100 (Terochem, Edmonton, Alberta, Canada); emulsions of LA and γ-linolenic acid (Sigma) were prepared by sonication in 1% Triton X-100 immediately before use. One of these compounds or an equivalent amount of 1% Triton X-100 alone was added to parsley cell cultures 5 d after subculture. Elicitor treatment of detached tobacco leaves was carried out as described previously (Douglas et al., 1991). Parsley and tobacco plants were treated with jasmonates and linolenic acid by spraying solutions of the test compounds or 1% Triton X-100 alone onto leaves until run-off occurred (MJ-treated plants were subsequently enclosed in a bell jar). Plants were kept at 28°C in constant light until harvest of leaf tissue. Parsley and tobacco were wounded by slicing detached leaves into 1- to 2-mm strips and incubation on filter paper moistened with Murashige-Skoog medium. After wounding, material from several leaves was pooled and then divided for harvesting at different time points. In some experiments, unwounded control experiments were carried out using detached leaves that were incubated as described above but not further wounded.

Treatment with nPG

Stock solutions of nPG (Sigma) were prepared in sterile 0.2 M potassium phosphate buffer, pH 7.0, as described by Staswick et al. (1991). Petioles of detached tobacco leaves were placed in nPG solution or in buffer alone for 12 h prior to wounding.

Furanocoumarin Analysis

Furanocoumarins were chloroform extracted from parsley suspension culture filtrates as described by Kombrink and Hahlbrock (1986) and quantified by reading the A_{320} of extracts. TLC of concentrated extracts was carried out as described previously (Kombrink and Hahlbrock, 1986).

RNA Isolation and Blot Hybridization

RNA was isolated from parsley cells and tobacco leaves by the method of Logemann et al. (1987). Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose affinity chromatography (Sambrook et al., 1989). Northern blot analysis was performed as previously described (Moniz de Sá et al., 1992) using 10 μg of total RNA per lane. To ensure evenness of loading, gels were stained with ethidium bromide prior to transfer. Slot blots were performed using a Schleicher & Schuell Minifold II apparatus. Hybridization probes were prepared from isolated plasmid inserts using a random primed labeling kit according to the recommendations of the manufacturer (Boehringer Mannheim). Parsley cDNA clones for the following genes were used to prepare probes: *4CL1* (Lozoya et al., 1988), *PAL1* (Lois et al., 1989), *BMT* (Hauffe, 1988; Hahlbrock and Scheel, 1989), *ELI3*, *ELI7*, and *HRGP* (Somssich et al., 1989; Trezzini et al., 1993), *TyrDC* (Somssich et al., 1989; Kawalleck et al., 1993a), and *ubi4* (Kawalleck et al., 1993b). The original designations of *TyrDC*, *HRGP*, and *ubi4* were *ELI5*, *ELI9*, and *CON2*, respectively (Somssich et al., 1989). For hybridization to tobacco RNA, probes were prepared from a potato *4CL* cDNA clone (St4CL, Becker-André et al., 1991), a tobacco *4CL* cDNA clone (Lee and Douglas, 1996), and a tomato cDNA clone encoding ubiquitin (a gift of Luca Comai, University of Washington, Seattle). *GUS* hybridization probes were prepared from *GUS* inserts isolated from pRT99-GUSJD, a plasmid vector containing the *GUS* gene behind multiple cloning sites (Schulze-Lefert et al., 1989). Hybridization and washing conditions were as described previously (Moniz de Sá et al., 1992); all washes were carried out at high stringency (0.2× SSC, 68°C) except those using a potato 4CL probe, St4CL, and GUS probes, which were carried out at low stringency (2× SSC, 68°C). All experiments were repeated at least once, with similar results.

RESULTS

Effect of Jasmonates on Gene Expression in Parsley

We chose *4CL* as a representative gene for dose-response experiments aimed at directly testing the activity of MJ, JA, and LA in inducing phenylpropanoid gene expression in suspension-cultured parsley cells. *4CL* transcript accumulation was measured using RNA hybridization and scanning densitometry. *4CL* hybridization intensities were normalized to signals obtained from duplicate blots hybridized to a probe for *ubi4*, a parsley polyubiquitin gene that has expression that is unaffected by elicitor (Kawalleck et al., 1993b). Figure 1 shows that 1.0 μM MJ induced significant *4CL* mRNA accumulation and that maximum induction of RNA accumulation occurred at 100 μM. JA was relatively more active at lower concentrations than was MJ (maximal activity was observed at 1.0 μM), and *4CL* mRNA accumulation was also strongly induced by the JA precursor LA at concentrations between 1.0 and 100 μM. To examine the specificity of

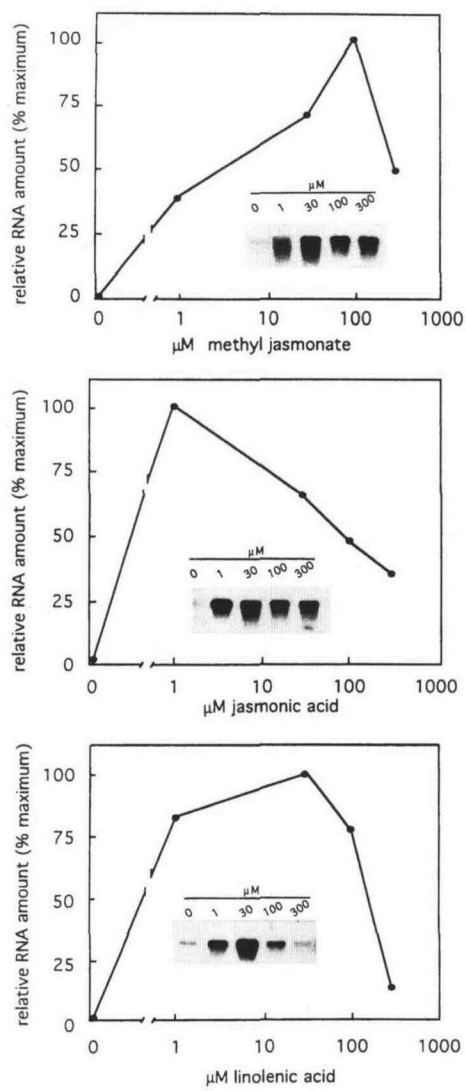


Figure 1. *4CL* mRNA accumulation in parsley suspension-cultured cells treated with MJ, JA, or LA. RNA was isolated from cells treated for 6 h with various concentrations of each compound in 1% Triton X-100 or with 1% Triton X-100 alone (0 μM). Duplicate northern blots were hybridized to parsley *4CL* and *ubi4* probes, and the autoradiographs were subjected to scanning densitometry. Relative *4CL* mRNA amounts were calculated after standardization to *ubi4* signal intensities. Insets show the northern blots hybridized to the *4CL* probe.

LA in activating *4CL* expression, cells were also treated with its isomer γ-linolenic acid, which does not enter the biosynthetic pathway to JA (Hamberg and Gardner, 1992). Over the range of concentrations used in this experiment, γ-linolenic acid was unable to induce *4CL* mRNA accumulation above control levels (data not shown). Thus, in addition to exogenously applied MJ and JA, endogenous JA made from LA may be effective in activating *4CL* gene expression in parsley cells.

If the ability of jasmonates to induce *4CL* mRNA accumulation is a reflection of their role as signaling intermediates in the response of parsley cells to elicitor, they would be expected to induce patterns of gene expression similar to those observed after elicitor treatment. We examined the accumulation of *PAL*, *4CL*, and *BMT* mRNAs in response to MJ and compared this with the well-characterized elicitor responsiveness of these genes in parsley cells (Hauffe, 1988; Lois et al., 1989; Lozoya et al., 1991). Figure 2 shows that treatment with 30 μM MJ induced the accumulation of *4CL* and *PAL* transcripts with kinetics similar to those in elicitor-treated cells but with a lower level of transcript accumulation. Activation of *BMT* transcript accumulation, an elicitor-specific response in parsley cells (Lozoya et al., 1991), appeared to occur more rapidly in response to MJ than to elicitor (compare 6-h points) and by 9 h reached a level higher than that seen after elicitor treatment. This suggests that MJ, like elicitor, may activate the coordinate and sequential expression of genes required for the biosynthesis of furanocoumarin phytoalexin biosynthesis in parsley cells. To further test this, the accumulation of furanocoumarins in parsley cell culture filtrates was determined 24 h after treatment with either MJ or elicitor. In the experiment shown in Figure 3, MJ induced furanocoumarin accumulation to a level severalfold above that found in control cells, but this level was about one-third of that induced by elicitor treatment. Similar results were found in repetitions of this experiment. TLC of the extracts shown in Figure 3 indicated that the furanocoumarins that accumulated in MJ-treated cells were identical with those in elicitor-treated cells (data not shown).

As another test of the relative responses of cultured parsley cells to elicitor and jasmonates, we examined the expression of *HRGP*, *ELI3*, *TyrDC*, and *ELI7* as representatives of a large set of non-phenylpropanoid defense-related genes transcriptionally activated following elicitor treatment (Somssich et al., 1989). Figure 4 shows that expression of all four genes was rapidly and strongly induced by elicitor treatment, with kinetics consistent with previous reports (Somssich et al., 1989). Treatment with 30 μM MJ strongly induced the accumulation of *HRGP*, *ELI3*, and *TyrDC* mRNAs but had no effect on *ELI7* transcript accumulation. The responsiveness of *HRGP*, *ELI3*, and *TyrDC* to MJ and elicitor treatments was generally similar, but *HRGP* mRNA accumulation appeared to be more rapidly induced by MJ and *TyrDC* mRNA accumulated to somewhat lower levels in MJ-treated cells.

To determine whether the response of elicitor-inducible genes to MJ is a phenomenon unique to cell cultures, we

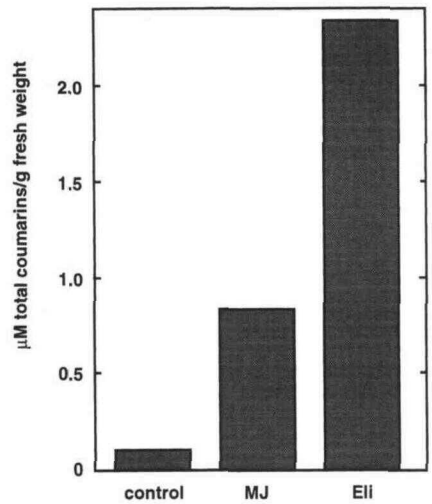
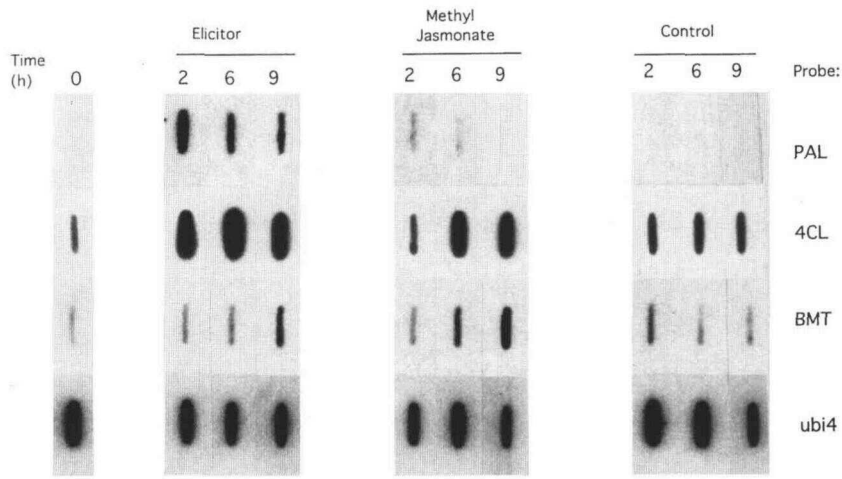


Figure 3. Accumulation of furanocoumarins in culture filtrates of parsley cells treated with MJ or Pmg (Eli). Parsley cell cultures were treated with either 30 μM MJ or 50 μg/mL Pmg; control cells were treated with 1% Triton X-100 alone. Furanocoumarins were extracted and quantified from culture filtrates 24 h after the start of treatments. Results shown are from a single experiment; replications of the experiment gave similar results.

Figure 2. Time course for the accumulation of mRNA specific to phenylpropanoid genes in parsley cells treated with either 50 μg/mL Pmg or 30 μM MJ; control cells were treated with 1% Triton X-100 alone. RNA was extracted from cells treated 0, 2, 6, or 9 h after treatment, and 2 μg was loaded onto duplicate slot blots that were hybridized to probes for parsley *PAL*, *4CL*, *BMT*, and *ubi4* (as a control for loading).



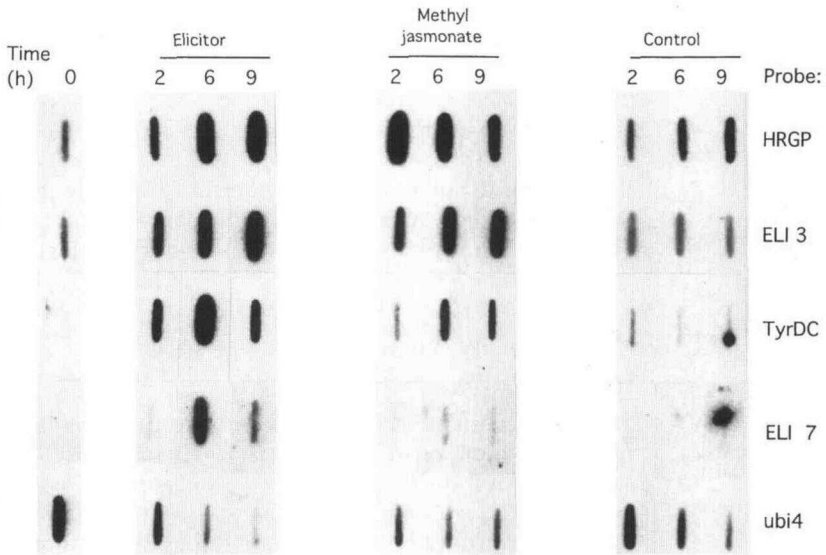


Figure 4. The effect of Pmg or MJ treatment on the expression of defense-related genes in parsley cells. Cells were treated as described in the legend to Figure 2. Duplicate slot blots of 2 μg of total RNA isolated from cells harvested at each time were hybridized to probes for parsley *HRGP*, *ELI3*, *TyrDC*, *ELI7*, and *ubi4*.

also tested their response to MJ in intact parsley plants. In parallel, we used parsley plants to determine whether expression of the non-phenylpropanoid elicitor-inducible genes is also wound inducible, as is *PAL* and *4CL* expression. Figure 5 shows hybridization of total RNA from wounded and MJ-treated parsley plants to probes for *PAL*, *4CL*, and other elicitor-inducible genes. *4CL* and *PAL* mRNAs accumulated above control levels by 6 h in MJ-treated plants, and elevated levels were still present 24 h after MJ treatment. As predicted, wounding rapidly induced *4CL* and *PAL* transcript accumulation, and increased mRNA levels were still present 24 h after wounding. Expression of *HRGP* and *ELI3* in intact plants was also strongly induced by MJ treatment, whereas the expression of *ELI7*, which was not MJ-inducible in cell cultures, was unaffected by MJ treatment of whole plants. However, expression of these genes was not activated by wounding. Despite its high level of induced expression in cell cultures (Fig. 4), expression of *TyrDC* was not detectable by RNA hybridization using total RNA from wounded, MJ-treated, or control plants (data not shown). Thus, in parsley MJ can activate expression of genes that are elicitor, pathogen, and wound inducible (*4CL* and *PAL*), as well as genes in which expression appears to be triggered specifically by elicitor and pathogen infection (*ELI3*, *TyrDC*, *HRGP*, and *BMT*).

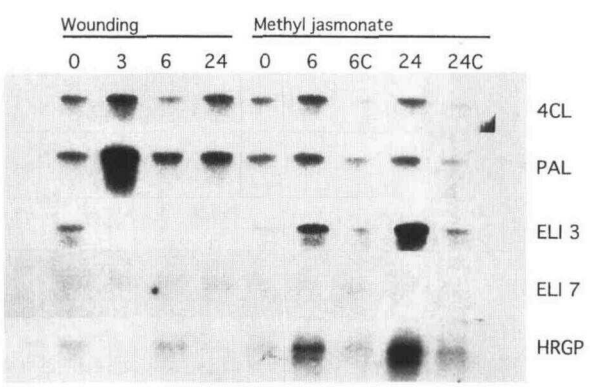


Figure 5. Expression of defense-related genes in wounded or MJ-treated parsley plants. Total RNA was extracted from wounded, excised leaves or leaves excised from whole plants sprayed with 1 mM MJ at various times (hours) after the onset of the treatments. Points at 0 h were from leaves excised from untreated plants. RNA samples from control plants (6C, 24C) were extracted from leaves excised from plants 6 and 24 h after spraying with 1% Triton X-100 alone. RNA samples (10 μg) were loaded on duplicate gels, and northern blots were hybridized to probes for the parsley genes indicated.

Wound- and MJ-Induced Expression of *4CL1-GUS* in Transgenic Tobacco

We used a transgenic tobacco system to determine whether the *4CL1* promoter directs responsiveness to jasmonates and to test further the potential role of jasmonates in wound-inducible *4CL1* expression. We have shown previously that expression of both endogenous tobacco *4CL* and a 597-bp *4CL1* promoter-*GUS* transgene is wound inducible (Douglas et al., 1991; Lee and Douglas, 1996) and that a 210-bp *4CL1* promoter-*GUS* fusion is similarly wound inducible (M. Ellard-Ivey and C.J. Douglas, unpublished data). Transgenic tobacco line 801–8, harboring a 597-bp *4CL1* promoter-*GUS* fusion (Hauffe et al., 1991), was used in dose-response experiments to assay the effectiveness of MJ, JA, and LA in activating *4CL1-GUS* expression. Plants were sprayed with solutions containing various concentrations of each compound, and *GUS* or tobacco *4CL* mRNA accumulation was measured by RNA hybridization 24 h after treatment. Signal intensities were estimated by scanning densitometry and were normalized to signals obtained by stripping blots and hybridizing to a probe for tomato ubiquitin. Figure 6 shows that accumulation of *GUS* transcripts from the introduced *4CL1-GUS* transgene was induced by each compound and that concentrations of 1 mM MJ, JA, and LA were most effective. Endogenous tobacco *4CL* gene expression, measured by hybridization to St4CL, was activated in a very similar

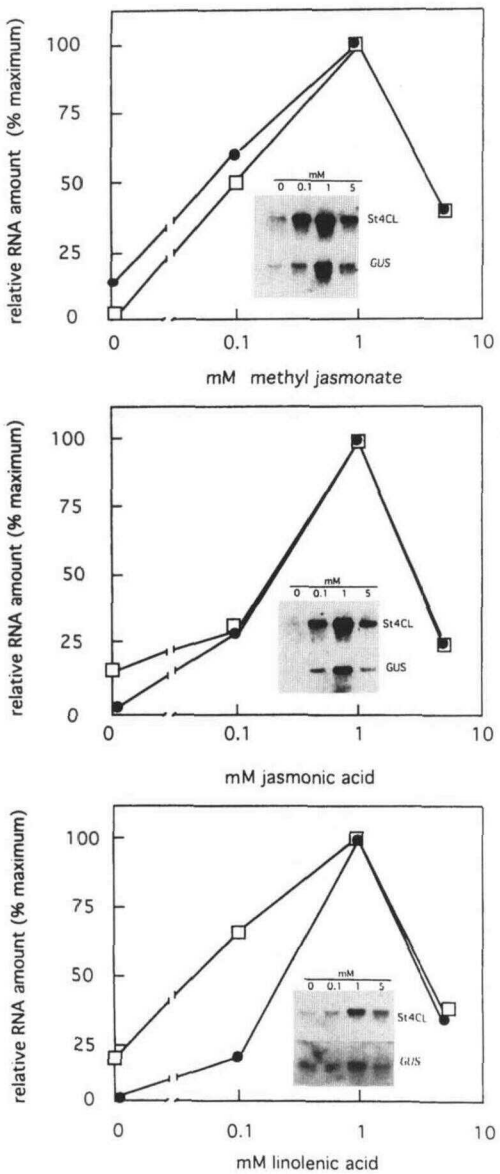


Figure 6. Accumulation of tobacco *4CL* and *GUS* mRNA in transgenic tobacco plants treated with MJ, JA, or LA. RNA was isolated from leaves of tobacco line 801–8 transgenic for a 597-bp *4CL1-GUS* fusion 24 h after spraying plants treated with various concentrations of each compound or after spraying with 1% Triton X-100 alone (0 μM). Duplicate northern blots were hybridized to potato *4CL* (St4CL) and *GUS* probes and then stripped and hybridized to a tomato *ubiquitin* probe. Autoradiographs were subjected to scanning densitometry. Relative *4CL* (●) and *GUS* (□) mRNA amounts were calculated after standardization to *ubiquitin* signal intensities. Insets show northern blots hybridized to potato *4CL* (St4CL) and *GUS* probes.

manner, suggesting that the *4CL1-GUS* transgene accurately reports the responsiveness of endogenous tobacco genes to jasmonates. When plants were treated with the same range of concentrations of γ-linolenic acid, neither *GUS* nor tobacco *4CL* expression increased above control levels after 24 h (data not shown). Further experiments showed that *GUS* expression was MJ inducible in additional transgenic tobacco lines expressing either the 597-bp *4CL1* promoter-*GUS* fusion or the 210-bp *4CL1* promoter-*GUS* fusion and that maximal *GUS* and tobacco *4CL* transcript accumulation occurred by 6 h after MJ treatment (data not shown). Thus, the *4CL1* promoter is strongly responsive to wounding, jasmonates, and the jasmonate precursor LA in transgenic tobacco. These results are consistent with a potential role for jasmonates as signaling intermediates for stress-activated phenylpropanoid gene expression and complement those results obtained in parsley.

Effect of the nPG Pretreatment on Stress-Induced Gene Expression

If endogenous JA, synthesized de novo from LA in response to wounding or elicitor treatment, is an intracellular signal required for the activation of parsley defense gene expression in response to these stresses, inhibitors of JA biosynthesis would be predicted to inhibit these responses by preventing the wound- or elicitor-stimulated biosynthesis of endogenous JA. The free radical scavenger nPG is a well-characterized lipoxygenase inhibitor. Because of its potency in inhibiting tobacco lipoxygenase (Fournier et al., 1993), an activity required for the de novo biosynthesis of JA from LA, we tested the ability of this compound to inhibit elicitor-activated gene expression in parsley cells and its ability to inhibit wound-activated gene expression in tobacco.

The effect of a 16-h nPG pretreatment on elicitor-induced accumulation of defense gene transcripts in parsley cells was examined (Fig. 7). RNA was isolated 6 h after the onset of elicitor or MJ treatments and at the same time from control cells. Treatment of cells with elicitor or MJ alone induced the accumulation of *PAL*, *4CL*, *TyrDC*, *ELI3*, and *HRGP* transcripts, whereas nPG treatment alone induced none of these (the apparent higher level of *ELI3* RNA in nPG-treated cells was not reproducibly observed). In the experiment shown, the levels of *4CL*, *PAL*, and *TyrDC* mRNAs were reduced by more than 3-fold in cells pre-

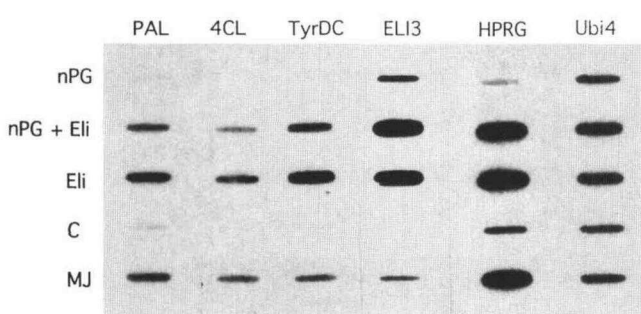


Figure 7. Effect of nPG on elicitor-stimulated accumulation of defense-related genes in parsley cell cultures. Cell cultures were pretreated for 16 h with 50 μM nPG and subsequently treated with 50 μg/mL Pmg (nPG + Eli) or left untreated (nPG). Alternatively, cultures were pretreated with buffer alone and subsequently treated with 50 μg/mL Pmg (Eli), 30 μg/mL MJ, or left untreated (C). Total RNA (2 μg) extracted from cells 6 h after treatments was loaded onto duplicate slot blots and hybridized to probes for parsley *PAL*, *4CL*, *TyrDC*, *ELI3*, or *ubi4*.

treated with nPG (nPG + Eli). In contrast, *HRGP* and *ELI3* were still fully responsive to elicitor treatment, and the expression of *ubi4* was unaffected by nPG pretreatment. In repetitions of this experiment, an approximately 10-fold decrease in *PAL* and *4CL* mRNA accumulation was sometimes observed in nPG-pretreated cells, whereas pretreatment with nPG had no effect on responsiveness to MJ (data not shown).

Finally, the effect of nPG on wound-inducible gene expression in tobacco was tested using transgenic line 801–8, in which the 597-bp *4CL1-GUS* transgene is both wound and jasmonate inducible. Excised leaves were treated for 10 h with buffer alone or with nPG and then wounded. Duplicate northern blots were hybridized to *GUS*, tobacco *4CL*, and tomato *ubiquitin* probes. Figure 8 shows the results of a representative experiment. In buffer-treated or 5 μM nPG-treated leaves, both *GUS* expression and tobacco *4CL* expression were wound inducible; however, in leaves pretreated with 50 μM nPG, the wound-inducible expression of both genes was abolished. Low levels of *GUS* and tobacco *4CL* transcripts, equivalent to those found in unwounded leaves, were still detectable in leaves pretreated with 50 μM nPG, and *ubiquitin* gene expression was unaffected by nPG pretreatment. Thus, at a concentration of 50 μM, nPG appears to specifically inhibit the responsiveness of the *4CL1-GUS* transgene and tobacco *4CL* to wounding.

DISCUSSION

Jasmonates may serve as signal compounds that mediate the activation of plant gene expression in response to stress (Staswick, 1992; Sembdner and Parthier, 1993; Farmer, 1994; Reinbothe et al., 1994). Because the expression of genes encoding enzymes of phenylpropanoid metabolism is both wound and elicitor inducible, insights into the potential role played by jasmonates in activating gene expression in response to these two kinds of stress may be gained by investigating the response of phenylpropanoid genes to jasmonates. In this study we used the parsley cell culture system and transgenic tobacco plants expressing parsley *4CL1-GUS* fusions to investigate the potential role played by jasmonates in mediating the wound- and elicitor-inducible expression of *4CL* and other parsley defense-related genes.

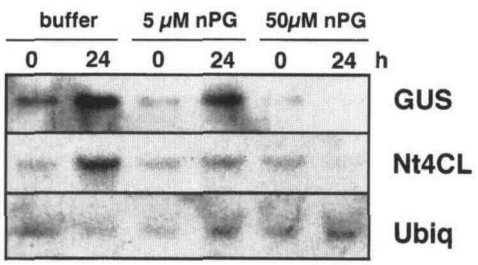


Figure 8. Effect of nPG pretreatment on wound-induced *GUS* and tobacco *4CL* mRNA accumulation in transgenic tobacco plants. Detached leaves from line 801–8 transgenic for a 597-bp *4CL1-GUS* fusion were pretreated for 16 h with buffer alone (buffer), 5 μM nPG, or 50 μM nPG and then either wounded and incubated for 24 h (24) or harvested without wounding (0). Duplicate northern blots of total RNA from each treatment were hybridized to probes for *GUS*, tobacco *4CL* (Nt4CL), or tomato *ubiquitin* (Ubiq).

In parsley cell cultures *4CL* expression was responsive both to jasmonates and to the jasmonate precursor LA in a dose-dependent manner (Fig. 1); a similar responsiveness of the *4CL1* promoter was observed in transgenic tobacco (Fig. 6). The activity of LA, but not its isomer γ-linolenic acid, suggests that LA may be converted into endogenous jasmonates via lipoxygenase activity (Farmer and Ryan, 1992), which subsequently activates *4CL* expression. Furthermore, expression of *PAL* and *BMT* was also activated in MJ-treated parsley cells, and MJ-activated *BMT* mRNA accumulation was delayed relative to that of *4CL* and *PAL*, which is typical of the elicitor response (Hauffe, 1988; Lozoya et al., 1991). Thus, genes encoding enzymes of phenylpropanoid metabolism are coordinately and sequentially activated by jasmonates. These results are in accordance with the observed activation of *4CL* and *PAL* mRNA accumulation in parsley cells by the jasmonate precursor phytodienoic acid (Dittrich et al., 1992) and the jasmonate-induced expression of *PAL* and *CHS* gene expression in other plants (Creelman et al., 1992; Gundlach et al., 1992). However, the MJ-stimulated accumulation of *PAL* and *4CL* transcripts that we observed was substantially greater than that reported following phytodienoic acid treatment of a parsley cell culture (Dittrich et al., 1992). It is difficult to compare our results with those, since that cell culture and its growth conditions were different from those used in our experiments, and only a single (10 μM) concentration of phytodienoic acid was used.

The activation of phenylpropanoid gene expression by jasmonates in parsley cells is consistent with arguments for a general role for jasmonates as signaling intermediates in the activation of cellular responses to elicitors (Gundlach et al., 1992; Mueller et al., 1993). Our observation that MJ treatment stimulated the accumulation of furanocoumarins (Fig. 3), parsley phytoalexins in which biosynthesis is activated specifically by elicitor treatment (Hahlbrock and Scheel, 1989), is also consistent with a potential role for jasmonates in elicitor-activated signaling pathways in parsley. However, concentrations of MJ that are optimal in activating *4CL* expression consistently activated the accumulation of furanocoumarins to levels only one-third of those observed following elicitor treatment, suggesting that jasmonates alone are unable to fully replace elicitor in activating this aspect of the elicitor response. Similarly, the timing and extent of defense gene mRNA accumulation induced by MJ were different from those induced by elicitor in some cases (Fig. 2 and 4). Strikingly, *ELI7*, a parsley gene that has expression that is strongly stimulated by elicitor treatment, was unresponsive to MJ treatment in both cell cultures and parsley plants (Figs. 4 and 5). Together, these results indicate that jasmonates can partially activate parsley responses to elicitor but are insufficient to fully activate these responses and do not completely mimic the effect of elicitor treatment on parsley cells. Thus, it is likely that signal transduction pathways independent of jasmonates are required to initiate and coordinate the full elicitor response in these cells.

Attenuation of furanocoumarin accumulation in parsley cells has been observed following simultaneous treatment with light and elicitor (Lozoya et al., 1991). MJ treatment has also been reported to stimulate flavonoid accumulation (Dittrich et al., 1992), which is normally a light-specific response in parsley cells (Hahlbrock and Scheel, 1989). Thus, the decreased levels of furanocoumarins in MJ-treated cells relative to elicitor-treated cells may reflect a similar phenomenon. However, we found that treatment of parsley cells with both MJ and elicitor had no effect on furanocoumarin accumulation relative to levels found in elicitor-treated cells (M. Ellard-Ivey and C. Douglas, unpublished data). Thus, unlike light, MJ does not repress this aspect of the elicitor response in parsley cells.

Since *4CL* and *PAL* are wound as well as elicitor inducible, the effect of MJ on gene expression in parsley cell cultures could be a reflection of its primary function as a wound-generated signaling molecule in addition to or instead of as a signaling intermediate in the elicitor response. Indeed, the strong jasmonate-induced expression of *4CL1-GUS* and tobacco *4CL* transgenic tobacco plants correlates with the wound inducibility of these genes in these lines. If this hypothesis were true, we predicted that all MJ-inducible parsley genes we tested would also be wound inducible. Although *4CL* and *PAL* were clearly both wound and MJ inducible in parsley plants (Fig. 5), and *ELI3* and *HRGP* were clearly MJ inducible, we found no evidence that *ELI3* and *HRGP* were also wound inducible. Thus, in parsley MJ responsiveness and wound responsiveness are not strictly correlated, suggesting that the functional role of MJ is not restricted to activating the expression of wound-inducible, defense-related genes.

Inhibitors of jasmonate biosynthesis have been used to demonstrate the requirement for endogenous jasmonate biosynthesis for wound-induced gene expression (Staswick et al., 1991; Peña-Cortes et al., 1993; Farmer et al., 1994). nPG, an inhibitor of lipoxygenases and/or cyclooxygenases, has been proposed to block the biosynthesis of the JA precursor phytodienoic acid (Peña-Cortés et al., 1993). nPG strongly inhibits the activity of a tobacco lipoxygenase that may be involved in JA biosynthesis from LA (Fournier et al., 1993) and has been shown to inhibit wound-induced expression of *pin* and *vsp* genes (Staswick et al., 1991; Peña-Cortes et al., 1993). In parsley cells nPG was partially effective in reducing the elicitor-inducible expression of *4CL*, *PAL*, and *TyrDC* but was ineffective in reducing *ELI3* or *HRGP* expression. Although we have no direct evidence that nPG blocks MJ biosynthesis in parsley cells, these results are consistent with the existence of signaling pathways in the elicitor response that are both dependent on and independent of jasmonates. If we assume that nPG treatments do affect jasmonate biosynthesis in parsley, a possible explanation for the moderate effect of nPG on the elicitor-inducible expression of some MJ-responsive genes (*4CL*, *PAL*, and *TyrDC*) and its ineffectiveness in altering the elicitor-inducible expression of others (*ELI3* and *HRGP*) is that jasmonate biosynthesis is not completely blocked and that endogenous jasmonate levels in nPG-treated cells are below a threshold required for activation of some genes but not others. Alternatively, some (or all) elicitor-inducible genes tested could be activated by multiple, branching signal transduction pathways, only one of which involves jasmonates or related octadecanoids.

In contrast to parsley cells, nPG had a strong inhibitory effect on wound-induced *4CL1-GUS* and *4CL* gene expression in tobacco (Fig. 8) but did not affect low levels of constitutive expression of these genes or constitutive expression of tobacco *ubiquitin* genes. If this inhibitory activity of nPG is due to blockage of wound-induced accumulation of endogenous jasmonates, these data suggest that jasmonates are required as signaling intermediates for the wound-induced expression of *4CL* and, possibly, other phenylpropanoid genes.

The ability of nPG to effectively inhibit plant oxygenases and thus biosynthesis of jasmonates via the octadecanoid pathway is related to its activity as an antioxidant, free radical scavenger (Staswick et al., 1991; Fournier et al., 1993). Activated oxygen species are produced following elicitor treatment of parsley cells (Nürnberger et al., 1994), and such species could play a role in elicitor-induced signal transduction (Apostol et al., 1989; Devlin and Gustine, 1992; Mehdy, 1994). Thus, an alternative explanation for the inhibitory effects of nPG on elicitor and wound stimulation is that such scavenging activity reduces the accumulation of activated oxygen species involved in signal transduction outside of the jasmonate biosynthetic pathway. However, there is currently no evidence that activated oxygen species are involved in elicitor-induced signal transduction in parsley cells (Nürnberger et al., 1994) or in the wound response.

Taken together, our experiments support a role for jasmonates in mediating some aspects of the elicitor response and in mediating the wound responsiveness of *4CL* but suggest that at least some of the responses to elicitor are mediated by signaling pathways not involving jasmonates. It is possible that multiply branched pathways involving different cellular intermediates, including but not restricted to jasmonates, emanate from the elicitor receptor (Mehdy, 1994). The exact role played by jasmonates in elicitor- and wound-induced signaling will await careful quantification of jasmonate levels, use of inhibitors specific to the octadecanoid pathway, and/or genetic manipulation of jasmonate biosynthesis.

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